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Characterization of CB_1 Cannabinoid Receptors Using Receptor Peptide Fragments and Site-Directed Antibodies

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ABSTRACT

The mechanism by which CB_1 cannabinoid receptors are coupled to the G_i/G_o class of G proteins was studied. A peptide representing the juxtamembrane carboxyl terminus robustly stimulated guanosine-5′-*O*-(3-thio)triphosphate binding. Peptides simulating subdomains of the third intracellular loop (IL3) activated minimally when present alone but produced additive effects when present in combination. Peptides representing the amino-side IL3 and the juxtamembrane carboxyl terminus autonomously inhibited adenylate cyclase, and this response was not significantly augmented or inhibited by peptides representing other intracellular domains. Site-directed antipeptide antibodies developed against the domains of the amino terminus, first extracellular loop, amino-side IL3, and juxtamembrane carboxyl terminus of CB_1 receptors failed to influence binding of [^{3}H]CP-55940. However, IgG raised against the amino-side IL3 diminished the agonist-dependent inhibition of adenylate cyclase. These experiments suggest that the juxtamembrane carboxyl terminus is critical for G protein activation by CB_1 cannabinoid receptors and that the amino-side IL3 also may interact with G_i proteins leading to inhibition of adenylate cyclase.

Δ^9-Tetrahydrocannabinol, the active compound in *Cannabis sativa*, and synthetic cannabimimetic compounds of cannabinoid, aminoalkylindole, and eicosanoid classes interact with cannabinoid receptors in the nervous system to produce their effects (for a review, see Howlett, 1995a). Stimulation of CB_1 cannabinoid receptors results in the pertussis toxin-sensitive inhibition of forskolin- or receptor-stimulated adenylate cyclase activity and regulation of ion channels, indicating that the cannabinoid receptor is coupled to G_i and G_o proteins (for a review, see Howlett, 1995b). The sequence of CB_1 cannabinoid receptors has been deduced for both rat and human species (Matsuda *et al*., 1990; Gerard *et al*., 1991).

In the current study, we sought to determine topographic regions of the CB_1 cannabinoid receptor that would be critical regions for agonist-induced activity. Peptides representing regions of the IL3 or the juxtamembrane carboxyl terminus were tested for their ability to compete for receptor/G protein interactions or to act autonomously to stimulate G proteins. Site-directed antibodies were generated against synthetic peptides representing the amino terminus, EL1, amino-side segment of the IL3, and juxtamembrane carboxyl-terminal regions of the rat brain CB_1 receptors. These antisera were tested for their ability to occlude ligand binding to the receptor or coupling of the receptor to G proteins in membrane preparations. Based on the findings of the peptide and antibody interactions, it was concluded that the juxtamembrane carboxyl terminus is critical for G protein activation by CB_1 cannabinoid receptors and that the amino-side IL3 also may interact with G_i proteins leading to inhibition of adenylate cyclase.

Materials and Methods

Peptides. Peptides (Table 1) CB1–14, CB174–188, CB301–317, and CB401–417 were synthesized using *t*-butyloxycarbonyl chemistry and purified by C-18 reverse-phase high performance liquid chromatography eluted with a gradient extending over 35 min from 0.1% trifluoroacetic acid to 80% acetonitrile plus 0.085% trifluoroacetic acid. Peptides CB316–327, CB329–344, and CB401–417 were synthesized by 9-fluorenylmethoxycarbonyl chemistry and purified by C-18 reverse-phase high performance liquid chromatography (Princeton Biomolecules, Columbus, OH). The amino acid sequence of peptide CB401–417 was verified by amino acid sequence analysis using automated Edman degradation and gas chromatography/mass spectometry. There is 100% identity in the amino acid sequences of these six peptides between rat and human CB_1 receptors (Matsuda *et al*., 1990; Gerard *et al*., 1991). CB1–14 is 64 amino acids proximal to the first of three potential amino-linked glycosylation sites on the

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ABBREVIATIONS: IL3, third intracellular loop of the G protein-coupled receptor; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate; DALN, desacetyllevonantradol; EL1, first extracellular loop of the G protein-coupled receptor; ELISA, enzyme-linked immunosorbent assay; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; KLH, keyhole limpet hemocyanin.

TABLE 1

Rat brain cannabinoid receptor peptide fragments and corresponding antisera

The peptide numbers correspond to inclusive amino acid residues of each sequence of the CB_1 cannabinoid receptor as reported by Matsuda *et al.* (1990). Amino acids in parentheses were added to peptide sequences to facilitate coupling peptides to protein carriers. For peptide CB401–417, the amino-terminal lysine added for coupling was not present in the peptide used for biochemical studies. The underlined amino acid replaced a cysteine in the sequence.

Peptide	Amino acid sequence	Location	Antisera
CB1–14	MKSILDGLADTTFR(C)	Amino terminus	Anti-CB1–14
CB174–188	(K)SFVDFHVFHRKDSPN	EL1	Anti-CB174–188
CB301–317	KAHSHAVRMIQRGTQKS	N-side IL3	Anti-CB301–317
CB316–327	KSIIIHTSEDGK	Central IL3	
CB329–344	QVTRPDQARMDIRLAK	C-side IL3	
CB401–417	(K)RSKDLRHAFRSMFPSSE	Carboxyl terminus	Anti-CB401–417

amino terminus, and therefore the epitope on the receptor is not likely to be obscured by the bulky oligosaccharides. The peptides have poor homology (0–30%) with related domains of the CB_2 receptor, with the exception of CB174–188 (EL1), which exhibits 64.3% identity and 5 consecutive amino acids.

Antibodies and immunoblots. Peptide CB1–14, modified by a cysteine at the carboxyl terminus (CB1–14), was conjugated at a 40-fold molar excess to sulfo-SMCC-KLH (Pierce Chemical, Rockford, IL). CB301–317 and lysine-modified CB174–188 and CB401–417 were conjugated to KLH in an 8–40-fold molar excess of peptides using glutaraldehyde (Song and Howlett, 1995). In CB401–417, Cys416 was replaced with serine to avoid potential artifactual disulfide bond formation. New Zealand White rabbits were injected intradermally with peptide-KLH conjugates (50 μg/rabbit), and antisera were screened by ELISA using peptide-BSA conjugates on Immulon 2 plates (Dynatech, Chantilly, VA). Plates were incubated and washed sequentially with antisera, goat anti-rabbit IgG conjugated with horseradish peroxidase (Pierce) (1:6,000), and 2,2-azino-di-(3-ethylbenzthiazoline)-6 sulfonate (Pierce) in 2.5 mM H_2O_2, 0.1% Tween 20, and 100 mM sodium acetate, pH 4.2, and the absorbance at 405 nm was determined with a Molecular Devices (Menlo Park, CA) plate reader. By ELISA, all antisera recognized their respective immunization peptides in the peptide-BSA conjugates but not unconjugated BSA or alternative peptide conjugates; the corresponding preimmune sera showed no significant reaction. Titers achieved were anti-CB1–14, 1:60,000; anti-CB174–188, 1:40,000; anti-CB301–317, 1:8,000; and anti-CB401–417, 1:2,300. Low-titer antisera may have been the result of a low molar ratio of peptides to KLH, which was limited by significant precipitation of conjugates having high molar ratios. IgG fractions for anti-CB301–317 and anti-CB401–417, and their preimmune sera were obtained by incubation with Protein A agarose (1 hr at 23°) according to the Pierce kit instructions. Protein concentrations were measured by absorbance at 280 nm.

Membrane preparation and signal transduction assays. Rat brain membranes were prepared from entire rat brain minus the brainstem as described previously (Devane *et al.*, 1988) with the addition of a protease inhibitor cocktail to the buffers (15 μg/ml benzamidine, 5 μg/ml leupeptin, 50 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.7 μg/ml pepstatin). N18TG2 cell membranes were prepared as described previously (Howlett, 1985).

Radioligand binding assay of the CB_1 cannabinoid receptor was performed and analyzed using [^{3}H]CP-55940 as described previously (Pinto *et al.*, 1994), with specific binding defined as [^{3}H]CP-55940 binding that was displaced by 100 nM DALN.

Adenylate cyclase assays were performed as described previously using N18TG2 neuroblastoma cell membranes (Howlett, 1985). Secretin (600 nM) was present as the hormonal stimulator, and Ro20–1724 (100 μM) was used as the phosphodiesterase inhibitor. Final concentrations of fatty acid-free BSA present in these assay mixtures were 0.16–0.21 mg/ml. The hormone-stimulated enzyme activities from this set of studies were 66.4 ± 3.67 (mean ± standard error, 13 experiments) pmol/min/mg of protein.

[^{35}S]GTPγS binding to rat brain membrane G proteins was performed according to a modification of the procedure of Lorenzen *et al.* (1993). Rat brain P2 membranes (5 μg of protein) were added to a reaction mixture containing 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 10 μM GDP, 0.375 nM [^{35}S]GTPγS, and the indicated concentrations of peptides or cannabinoid ligands (100 μl final volume). Binding of [^{35}S]GTPγS (30° for 60 min) was stopped by rapid filtration over GF/B (Whatman, Clifton, NJ) filters using a TOMTEC (Orange, CT) Harvester, and radioactivity was quantified using a 1205 Betaplate counter (Wallac, Gaithersburg, MD). Nonspecific binding was calculated as binding that could not be displaced by 100 μM GTPγS. Data were calculated as pmol of [^{35}S]GTPγS bound/mg of protein from an average of four replicates. Background values averaged 623 ± 68 fmol/mg (mean ± standard error, six experiments), and values stimulated by 1 μM DALN averaged 1150 ± 100 fmol/mg (six experiments).

Results

Determination of Functional Domains on Rat Brain Cannabinoid Receptors

Agonist binding sites. All study antisera specifically recognized a band with an apparent molecular mass of 64 ± 1.0 kDa (13 experiments), whereas preimmune sera did not (Song and Howlett, 1995; data not shown). The 64-kDa band is believed to be CB_1 cannabinoid receptors with carbohydrates at two glycosylation sites (Song and Howlett, 1995). To determine whether the domains of the receptor occluded by the antibodies overlap regions important for agonist ligand binding, rat brain membrane preparations were preincubated with each of the four antisera, and specific binding of [^{3}H]CP-55940 was determined. Compared with the effects of cognate preimmune sera, none of the antisera interfered with ligand binding to rat brain cannabinoid receptors (Table 2). The more concentrated antisera and preimmune sera (1:20 dilution) both attenuated the specific binding of [^{3}H]CP-55940, probably as the result of an interaction of serum lipoproteins and albumin with radioligand. Serum lipoprotein and albumin interactions with cannabinoid ligands have been reported previously (Wahlqvist *et al.*, 1970; Widman *et al.*, 1974; Poddar *et al.*, 1988) at concentrations equal to or greater than present in the assay mixture (1.2 mg of protein/ml for 1:20, 4.8 mg of protein/ml for 1:5 in Table 3).

Sites coupling to G proteins. Receptor peptide fragments and site-directed antibodies were used to investigate domains of the CB_1 receptor important for receptor/G protein interaction. None of the receptor peptide fragments (at 100 μM) was able to compete for DALN-induced receptor/G protein interactions as determined in either [^{35}S]GTPγS binding or adenylate cyclase assays (see Figs. 3 and 6). To determine whether receptor peptide fragments could autonomously activate G_i and G_o, these peptides were added with membranes directly into the incubation mixture in the absence of a stim-

TABLE 2

The effect of site-directed antisera on agonist ligand binding to rat brain cannabinoid receptors

Rat brain P2 membrane preparations were incubated with no sera, antisera, or preimmune sera at 4° for 4 hr before the addition (without washing) to the ligand binding reaction mixtures at 0.4 times the final assay volume. Under the conditions of the assay, the specific binding activity of 110 pM [^{3}H]CP-55940 in the absence of serum was 56.2 ± 4.3 and 61.2 ± 0.6 fmol/mg of protein for experiments A and B, respectively. These values represented ~70% of the total binding at this concentration of radioligand and membrane fraction. The data shown are the mean ± standard error from two experiments, each with triplicate determinations. There was no significant difference ($p < 0.05$) when the effect of antisera was compared with that of preimmune sera using a paired Student's t test.

Antibody	Serum dilution	Specific binding	
		Percent of total binding	Normalized to control
		%	
Experiment A			
None		69.9 ± 0.6	100.0
Anti-CB1–14	1:100	65.4 ± 0.5	93.5
	1:20	60.5 ± 0.3	86.5
Preimmune CB1–14	1:100	74.7 ± 2.1	106.8
	1:20	62.3 ± 1.5	89.1
Anti-CB174–188	1:100	72.8 ± 3.2	104.1
	1:20	64.2 ± 4.1	91.8
Preimmune CB174–188	1:100	72.0 ± 4.2	103.0
	1:20	60.7 ± 0.6	86.8
Experiment B			
None		68.2 ± 2.5	100.0
Anti-CB301–317	1:100	62.8 ± 4.2	92.1
	1:20	44.1 ± 6.7	64.6
Preimmune-CB301–317	1:100	67.8 ± 6.5	99.4
	1:20	45.1 ± 4.9	66.1
Anti-CB401–417	1:100	62.8 ± 4.2	92.1
	1:20	44.3 ± 3.3	64.9
Preimmune-CB401–417	1:100	70.4 ± 0.8	103.2
	1:20	51.9 ± 3.2	76.1

ulatory cannabimimetic agonist, and signal transduction activity was determined. Fig. 1 shows peptides tested for their ability to stimulate [^{35}S]GTPγS binding to G proteins in rat brain membrane preparations. The peptides representing the extracellular amino terminus and EL1 produced a background response in the assays. This was expected because these peptides represent domains of the receptor that would interact with neither the ligand nor the intracellular effector proteins. CB401–417 promoted a robust stimulation of [^{35}S]GTPγS binding to G proteins. When tested individually, CB301–317, CB316–327, and CB329–344, components of IL3, produced only background stimulation of [^{35}S]GTPγS binding. However, when present in combination, peptides representing the three segments of IL3 seemed to produce an additive stimulation (Fig. 2). This response was not simply due to the presence of a 3-fold greater concentration of peptide because a 300 μM concentration of either of the peptides alone produced responses that were not different from responses to the 100 μM concentrations (data not shown). These findings could indicate the necessity for some conformational interaction of all three subdomains to select for and activate G proteins. Alternatively, each subdomain might have the ability to select and activate one of multiple G protein subtypes, and the additivity represents multiple populations of G proteins. Combinations of IL3 component peptides with CB401–417 failed to significantly augment or inhibit the [^{35}S]GTPγS binding promoted by CB401–417 alone. Thus, activation by the juxtamembrane carboxyl-terminal domain seems to be the dominant influence on G protein activation, but the regions comprising IL3 also seem to exert a regulatory influence.

TABLE 3

The effects of site-directed antisera on DALN-induced inhibition of adenylate cyclase

N18TG2 cell membrane preparations were preincubated with diluted preimmune sera or antisera at 4° for 4 hr before the addition to the adenylate cyclase assay mixture. Data were normalized for each experiment so the inhibition by 100 nM DALN in the absence of either antiserum or preimmune serum was 100% inhibition. Data are expressed as the mean ± standard error of two to four experiments, each with three determinations.

	Antisera dilution	Adenylate cyclase inhibition	Adenylate cyclase inhibition normalized to control
		%	
Experiment A			
None		32.2 ± 4.1	100.0
Anti-CB1–14 (n = 2)	1:100	29.0 ± 7.7	90.1
	1:20	29.7 ± 4.5	92.3
	1:5	19.4 ± 5.6	60.3
Preimmune CB1–14	1:100	30.3 ± 4.0	94.1
	1:20	28.6 ± 6.9	88.8
	1:5	16.9 ± 5.0	52.5
Anti-CB174–188 (n = 2)	1:100	33.7 ± 2.6	104.6
	1:20	27.7 ± 9.6	86.0
	1:5	7.5 ± 4.9	23.3
Preimmune CB174–188	1:100	35.9 ± 5.4	111.5
	1:20	25.0 ± 3.4	77.6
	1:5	6.1 ± 2.3	18.9
Experiment B			
None		33.2 ± 1.2	100.0
Anti-CB301–317 (n = 4)	1:100	32.1 ± 5.2	96.7
	1:20	14.3 ± 10.7	43.1
	1:5	7.1 ± 3.7[a]	21.2
Preimmune CB301–317	1:100	32.9 ± 5.9	99.1
	1:20	23.7 ± 3.1	71.4
	1:5	21.1 ± 9.3	63.5
Anti-CB401–417 (n = 3)	1:100	34.8 ± 1.4	107.4
	1:20	29.4 ± 1.5	90.7
	1:5	10.8 ± 4.5	33.3
Preimmune CB401–417	1:100	32.4 ± 2.6	100.6
	1:20	26.9 ± 2.1	83.0
	1:5	12.2 ± 4.5	37.6

[a] Significantly lower than preimmune serum control at $p < 0.05$ using paired Student's one-tailed t test.

The response to peptides was additive with agonist-dependent stimulation by DALN (Fig. 3). This might suggest that the agonist/CB$_1$ receptor complex is a limiting factor in G protein stimulation in rat brain homogenates. An explana-

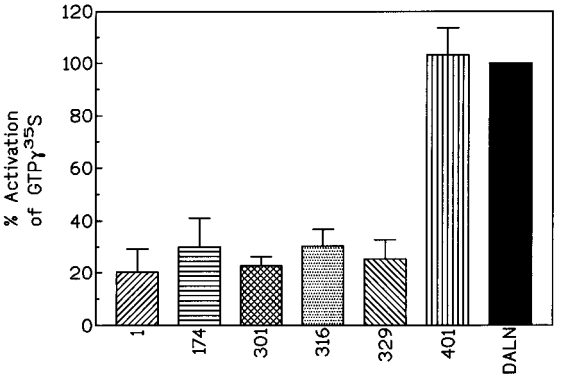

Fig. 1. Effect of CB$_1$ receptor peptide fragments on [^{35}S]GTPγS binding to rat brain membrane G proteins. Peptides (100 μM) were included in the incubation mixture without pretreatment, and accumulation of [^{35}S]GTPγS was determined. Data are presented as activation promoted by the peptide expressed as a percentage of the response to 1 μM DALN (i.e., stimulated by agonist/receptor complex), where 0% is the [^{35}S]GTPγS binding in the absence of hormonal or peptide stimulators. *Error bars*, standard error from three to five experiments for each peptide. Data were analyzed by analysis of variance followed by Tukey's *post hoc* test, and no significant differences ($p < 0.05$) were noted between peptides except for CB401–417, which differed from all others.

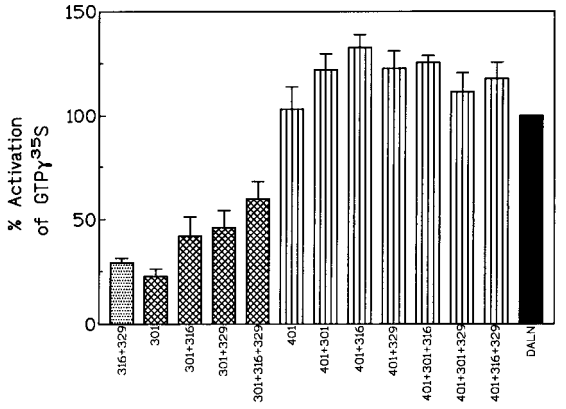

Fig. 2. Effects of combinations of CB_1 receptor peptide fragments on [^{35}S]GTPγS binding to rat brain membrane G proteins. Peptides (100 μM) were included in the incubation mixture in the indicated combinations and are presented as percentage of the response to 1 μM DALN (mean ± standard error) from three to five experiments for each peptide combination. Data were analyzed by analysis of variance followed by Tukey's *post hoc* test. Peptide combinations containing CB301–317 (▨) were not different from each other except for the combination of CB301–317 plus CB316–327 plus CB329–344, which differed from CB301–317 and from CB316–327 plus CB329–344 ($p < 0.05$). Peptide combinations containing CB401–417 (▥) were not different from each other at the ($p < 0.05$) level of significance.

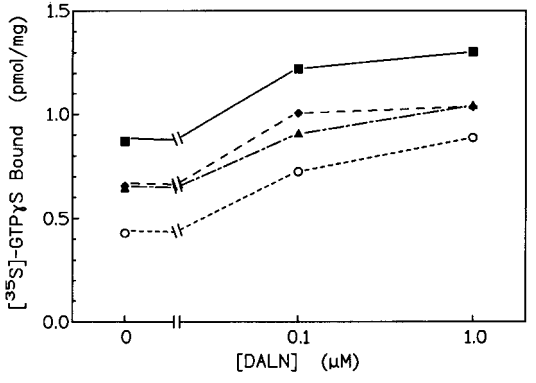

Fig. 3. Additivity of responses to peptides with that of the cannabinoid agonist-stimulated receptor. DALN was absent or present at the indicated concentrations in the presence of vehicle (○) or 100 μM CB316–327 (▲), CB329–344 (♦), or CB401–417 (■). Data are expressed as pmol of [^{35}S]GTPγS/mg of protein specifically bound. This is a single representative experiment.

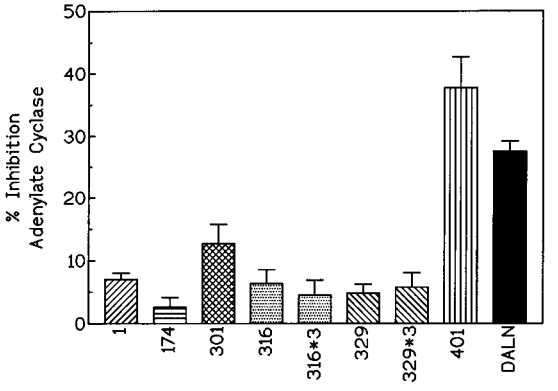

Fig. 4. Effect of CB_1 receptor peptide fragments on inhibition of adenylate cyclase activity. Peptides (100 μM, except *3 denotes 300 μM) are denoted by the number corresponding to the first amino acid of the peptide in the rat CB_1 receptor sequence (see Table 1). Data are presented as the percentage inhibition promoted by the presence of peptide (mean ± standard error) from three to seven experiments for each peptide. Data were analyzed by analysis of variance followed by Tukey's *post hoc* test. CB401–417 was significantly different from all other peptides ($p < 0.05$).

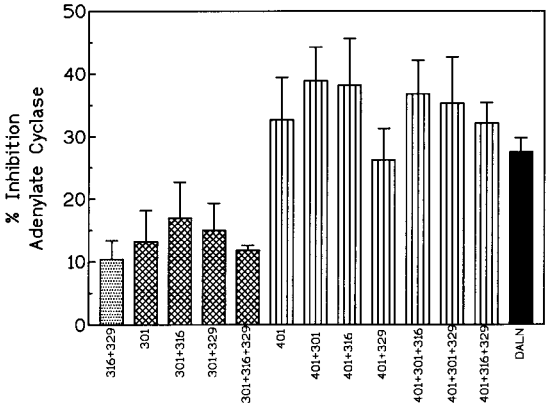

Fig. 5. Effects of combinations of CB_1 receptor peptide fragments on inhibition of adenylate cyclase activity. Peptides (100 μM) were included in the incubation mixture in the indicated combinations and are presented as the percentage inhibition promoted by the presence of peptides (mean ± standard error) from three to six experiments for each peptide. Data were analyzed by analysis of variance followed by Tukey's *post hoc* test. Peptide combinations within the groups containing CB301–317 (▨) or CB401–417 (▥) were not different from each other ($p < 0.05$).

tion for the additivity could be that the peptide fragments were activating G proteins that were not associated with agonist/receptor complexes. One could hypothesize that these G proteins would be of the $G_{i/o}$ class; however, the activation of other G protein classes cannot be dismissed.

Cannabinoid receptor-stimulated [^{35}S]GTPγS binding in rat brain homogenates would be likely to represent predominantly G_o (Garibay *et al.*, 1991). To determine whether peptide fragments of the CB_1 receptor could autonomously activate G_i, these peptides were added directly to the adenylate cyclase incubation mixture, and enzyme activity was measured in the absence of an agonist (Fig. 4). The peptides representing the extracellular amino terminus and EL1 produced a minimal response in the adenylate cyclase assays. The juxtamembrane carboxyl terminus robustly promoted inhibition of adenylate cyclase, with an efficacy that generally exceeded that of cannabinoid agonist/receptor-stimulated activity. The amino-side IL3 also inhibited adenylate cyclase, but the extent was limited to only ~40% of that promoted by the agonist/receptor complex. The magnitude of the response was not increased when 300 μM peptide was used (data not shown). The peptide fragments representing the central IL3 and carboxyl-side IL3, even at 300 μM, failed to evoke a response greater than those of the two extracellular domain fragments.

Combinations of peptides were tested to determine whether discrete CB_1 receptor domains could interact with each other to promote G_i activation. No synergism was observed on the addition of peptides representing the central and/or carboxyl-side regions of IL3 to the amino-side IL3 peptide (Fig. 5). Peptide combinations comprising IL3 failed to significantly alter the response to the juxtamembrane carboxyl-terminal domain peptide. Thus, the dominant activating effect of the amino-side carboxyl-terminal domain was neither attenuated nor augmented by the presence of any of the subdomains of IL3.

The cannabinoid agonist DALN inhibited adenylate cy-

clase activity with an EC_{50} value of 31 nM (confidence interval, 16–64 nM) and an asymptotic approach to a maximal inhibition of 40% of the hormone-stimulated activity in these experiments. Peptide CB301–317 alone inhibited adenylate cyclase activity by 15% and produced a less-than-additive response with increasing concentrations of DALN (Fig. 6). In the presence of peptide CB301–317, the EC_{50} value for DALN was 9.5 nM (confidence interval, 2–47 nM) and approached a maximal inhibition of 40% of the hormone-stimulated activity. Thus, there seemed to be a limitation in the amount of G_i activated in the presence of this peptide, and the response to agonist/receptor complex seemed to supersede that of the receptor fragment.

Peptide CB401–417 alone produced a robust inhibition of adenylate cyclase (32%) at 100 μM, a concentration that produced the maximum response (Mukhopadhyah S and Howlett AC, unpublished observations). This concentration of peptide CB401–417 produced additive effects with the agonist/receptor complex throughout the entire dose-response range for the agonist. In the presence of CB401–417, the EC_{50} value for DALN was 19 nM (confidence interval, 4–90 nM) and approached a maximal inhibition of 65% of the hormone-stimulated activity. Analysis of the curves indicates that the small decreases in EC_{50} values in the presence of peptides are not statistically significant. The increase in efficacy produced by peptide CB401–417 suggests a greater number of G_i proteins are activated in the presence of the receptor peptide fragment than are activated by the agonist/receptor complex alone. This suggests that the population of G_i proteins available for interaction with the peptide is distinct from the population of G_i proteins coupled to CB_1 receptors. The observations that the actions of hormone-stimulated receptor and peptide CB401–417 are neither synergistic nor competitive, and that the peptide did not alter the affinity of the hormone for the receptor suggests the peptide does not affect receptor/G protein coupling.

Agonist-induced inhibition of adenylate cyclase was assayed in N18TG2 membranes that had been preincubated with each of the four site-directed antisera or preimmune sera. Anti-CB1–14 and anti-CB174–188, antibodies that would occlude the amino terminus and EL1 of CB_1 receptors, each failed to alter the DALN-induced inhibition compared with the effect of the cognate preimmune sera (Table 3). Preincubation of membranes with antiserum against IL3 (anti-CB301–317) resulted in a dose-dependent attenuation of the DALN-induced inhibition of adenylate cyclase compared with the effect of cognate preimmune serum. The antiserum against the amino-side carboxyl terminus (anti-CB401–417) did not inhibit the effect of DALN on adenylate cyclase. The influence of the preimmune sera at low dilutions suggests interference that might be eliminated by isolating the IgG fraction by Protein A chromatography. The normalized DALN-induced inhibition of adenylate cyclase in the presence of IgG fractions of anti-CB301–317 versus cognate preimmune serum was 68.4% versus 93.2% at a low concentration of IgG (0.016 mg/ml) and 50.5% versus 85.5% at a high concentration (0.163 mg/ml) (Fig. 7). The IgG of anti-CB401–417, against the amino-side carboxyl terminus, failed to block the DALN response (Fig. 7). These data suggest that the CB_1 receptor IL3 may be involved in G_i/receptor coupling. However, the lack of response to the IgG of anti-CB401–417 cannot necessarily be interpreted to mean that this region fails to be involved in G_i/receptor coupling. The lack of response may simply be due to a poor affinity that the antibody may have for the receptor, resulting in the inability of the antibody to compete with a receptor/G protein interaction with relatively greater affinity.

Discussion

In the current report, we describe peptide fragments of the CB_1 cannabinoid receptor and four site-directed anti-peptide antisera against CB_1 receptors and the use of these tools to study functional domains of brain cannabinoid receptors. An important finding from these studies is that the juxtamembrane carboxyl-terminal domain peptide of the CB_1 cannabinoid receptor is a highly effective activator of $G_{i/o}$ proteins. Interaction of this receptor domain with G proteins has been reported for several other G protein-coupled receptors, including rhodopsin coupling to transducin as indicated by studies using synthetic peptides (Takemoto *et al.*, 1985; Phillips and Cerione, 1994) and alanine scanning mutagenesis (Osawa and Weiss, 1994). The amino-side carboxyl-terminal

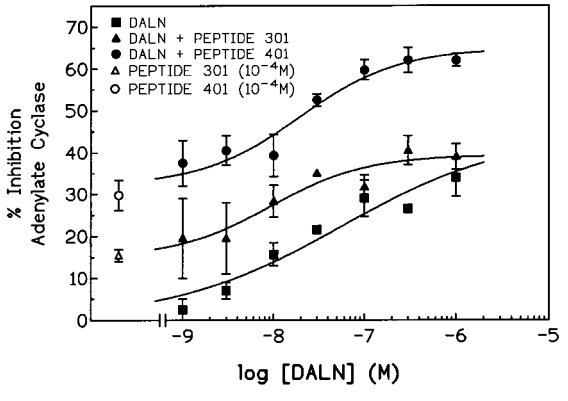

Fig. 6. Additivity between activating peptides and agonist/receptor complex for G_i activation. Peptides CB301–317 and CB401–417 (100 μM) were included in the incubation mixture in the presence or absence of increasing concentrations of the cannabinoid agonist DALN. Data are mean ± standard error from three experiments, and the curves were analyzed using GraphPAD InPlot.

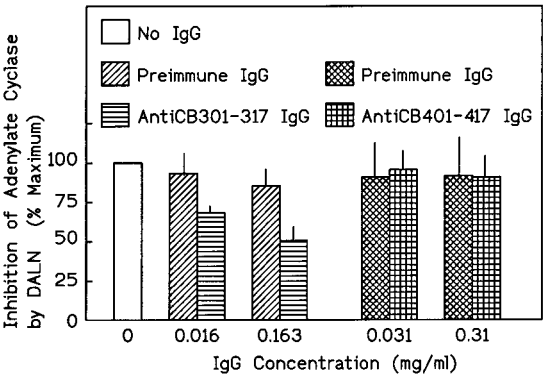

Fig. 7. DALN-induced inhibition of adenylate cyclase in the presence of IgG fractions of anti-CB301–317 and anti-CB401–417. The IgG fractions derived from the two antisera or their respective preimmune sera were preincubated with the N18TG2 cell membrane at 4° for 4 hr before direct addition to the adenylate cyclase assay mixture at 0.4 times the final assay volume. The inhibition of cAMP synthesis by DALN in the absence of any serum was 44%, and this value was normalized to be 100%. *Error bars*, mean ± standard error from triplicate determinations. Data shown are representative of four experiments.

region seemed to be important for β_2-adrenergic receptor coupling to G proteins in mutation studies (O'Dowd *et al*., 1988). A juxtamembrane carboxyl-terminal peptide from the N-formyl peptide receptor interacted with a carboxyl-terminal region of α_i in ELISA studies, and that peptide also disrupted the receptor/G protein complex in detergent solution (Schreiber *et al*., 1994). A major difference between the results reported here for the CB_1 receptor compared with other G protein-coupled receptors is that activation of a G protein by the carboxyl terminus has not yet been reported for other receptor types.

The CB_1 receptor proximal amino-side IL3 peptide is able to stimulate G_i activation, leading to inhibition of adenylate cyclase; however, this peptide does not exhibit as great an efficacy as the juxtamembrane carboxyl-terminal peptide. Another difference between the actions of these two peptide segments is that the amino-side IL3 peptide alone does not promote the activation of G proteins in brain membranes (predominantly G_o). Perhaps the IL3 receptor region facilitates G protein activation secondarily to or only in combination with a more effective stimulus.

For a number of G protein-coupled receptors, the carboxyl-side IL3 domain has been proposed to serve as a G protein recognition or coupling region (Dixon *et al*., 1987; O'Dowd *et al*., 1988; Wade *et al*., 1994; Yamada *et al*., 1994; Liu *et al*., 1995; Shi *et al*., 1995), although the data presented here do not support this role for the carboxyl-side IL3 of the CB_1 receptor. Evidence exists for a role for the carboxyl-side IL3 domain of G protein-coupled receptors in suppressing or restricting the G protein interaction in that point mutations in the carboxyl-side IL3 domain of the α_{1B}-adrenergic receptor (Cotecchia *et al*., 1990; Ren *et al*., 1993), α_{2A}-adrenergic receptor (Ren *et al*., 1993), and muscarinic acetylcholine (Hogger *et al*., 1995) receptors result in constitutively active receptors. When the carboxyl-side IL3 peptide of the CB_1 receptor was included in combination with the peptides that activate G_i and G_o, it failed to suppress activation. This evidence argues against a model in which this domain of the CB_1 receptor would suppress activity by associating with and thereby occluding a domain that activates G proteins.

For the CB_1 receptor, the combination of subdomains of IL3 seems to be necessary to observe activation of G proteins in rat brain membranes. This could result from a summation of the activation of multiple G protein subtypes, each activated by a different receptor domain. Alternatively, a G protein may possess multiple sites of interaction with the receptor, and the combination of peptides may trigger recognition and activation at these distinct sites. The finding that multiple patches on the receptor facilitate interaction with the G protein has been reported for other G protein-coupled receptors, including the IL2 and IL3 regions of the muscarinic acetylcholine receptor in chimeric mutant studies (Wong *et al*., 1990); IL2, IL3, and a region of the carboxyl terminus distal to the cysteine-palmitate anchor of rhodopsin in studies of peptides and peptide-directed antisera (Weiss *et al*., 1988; Konig *et al*., 1989); IL2, amino-side IL3, and amino-side IL4 of the β-adrenergic receptor in peptide studies (Munch *et al*., 1991); and amino- and carboxyl-side IL3 of the α_2-adrenergic receptor in studies of homodimers and heterodimers of peptides (Dalman and Neubig, 1991; Wade *et al*., 1994). Recognition sites on α- and β_2-adrenergic and muscarinic acetylcholine receptors have been demonstrated that promote coupling but fail to activate G proteins autonomously (Hausdorff *et al*., 1990; Luttrell *et al*., 1993; Hawes *et al*., 1994). For the β_2-adrenergic receptor, deletion mutation studies implicate a seven-amino acid segment in the carboxyl-side IL3 in transmitting the agonist-induced stimulatory signal to G_s and that this region is distinct from domains that function to promote the ternary complex (Hausdorff *et al*., 1990). Peptides from the amino-side IL3 and carboxyl-side IL3 of the β_2-adrenergic receptor activate G_s in phospholipid vesicle GTPase and GTPγS binding assays (Cheung *et al*., 1991). For the α_2-adrenergic receptor, IL2 or the carboxyl-side IL3 peptides reduced high affinity agonist binding, but only the latter could stimulate GTPase activity (Dalman and Neubig, 1991). These findings favor a mode of cooperativity between different domains of G protein-coupled receptors to activate G proteins.

The CB_1 carboxyl-terminal peptide activates G proteins in a manner that is additive with that of cannabinoid receptor-stimulated G protein activation. The CB_1 receptor/G protein complex seems to be stable in the absence of agonist in CHAPS-solubilized preparations (Houston and Howlett, 1993). The peptides seem to be activating a population of G proteins that are not coupled to the cannabinoid receptor. This differs from observations made for several other G protein-coupled receptors for which peptides compete for the receptor/G protein interaction (Konig *et al*., 1989; Phillips and Cerione, 1994; Schreiber *et al*., 1994; Wade *et al*., 1994).

The CB_1 receptor/G protein interaction could be disrupted by the antiserum or IgG fraction against the proximal segment of IL3. This finding is consistent with antibody competition for a G protein association site on the receptor. A similar disruption was reported for the D_2 receptor, in which an antibody against an IL3 peptide was able to disrupt the ternary complex (Boundy *et al*., 1993). The incomplete block of the cannabinoid inhibition of adenylate cyclase may suggest that the G_i protein interacts with multiple intracellular domains of the receptor that also may stabilize the receptor/G protein association and that the antibody inefficiently dislodges the G protein.

No effect of the antibody to the juxtamembrane carboxyl-terminal of the CB_1 receptor was observed when tested on cannabinoid-mediated inhibition of adenylate cyclase. If the juxtamembrane region of the CB_1 receptor association with the G protein is of high affinity, then an antibody may not be able to disrupt that preformed interaction. Alternatively, the CB_1 receptor/G protein interaction may occlude the juxtamembrane carboxyl-terminal domain of the receptor. This was the explanation for the failure of a carboxyl-terminal $\alpha_{i/o}$ antibody to interact with the α_2-adrenergic receptor/G protein ternary complex in the presence of agonist (Okuma and Reisine, 1992).

The current results have provided some insight into the nature of the CB_1 receptor/G protein interaction leading to G protein activation and signal transduction. Future studies will investigate the selectivity for G protein subtypes and the role of the α and $\beta\gamma$ subunits in the interaction with subdomains of the CB_1 receptor. Other studies will investigate the properties of the CB_1 receptor carboxyl-terminal juxtamembrane domain that regulate activation of G proteins.

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